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Demographic variation in bone-marrow derived mesenchymal stem cell analytes

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DEMOGRAPHIC VARIATION IN BONE-MARROW DERIVED MESENCHYMAL STEM CELL ANALYTES

by

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DEMOGRAPHIC VARIATION IN BONE-MARROW DERIVED MESENCHYMAL STEM CELL ANALYTES

MARGARET DUNLAP

ABSTRACT

Osteoporosis is a systemic skeletal disease that affects millions of people worldwide. There are many possible etiologies for osteoporosis, including inherent variables like genetics and sex, and lifestyle variables like diet and exercise. Characterized by low bone mass and increased fracture risk, the disease places a burden on both the patients and the healthcare industry. Therefore, it is vital that research determine the mechanisms by which the risk factors affect BMD so that better diagnosis and treatment options may be developed. The purpose of this study was to examine the relationship between various osteoporosis risk factors and biochemical markers of osteogenic cell activity derived from bone-marrow MSCs. It was hypothesized postmenopausal white women, having the greatest risk for osteoporosis, would have elevated hydroxyproline and decreased ALP, indicative of greater bone resorption. Acetabular reamings were collected from 26 patients (15 males and 11 females) undergoing total hip arthroplasty at Boston Medical Center. MSCs from the reamings were plated and underwent osteoinduction into osteoblasts. The cells were then harvested and assayed for various indicators of cell growth and bone cell activity, such as DNA, ALP, and hydroxyproline. Our hypothesis was generally supported in that postmenopausal white women did have less ALP, an indicator of bone deposition, than premenopausal women and postmenopausal African

American women. Additional findings and directions for future studies are further discussed in this paper.

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LIST OF ABBREVIATIONS

ALP..... Alkaline Phosphatase

α-MEM.....Minimum Essential Medium-alpha

Anti-anti..... Antibiotic-antimycotic

ARS ..... Alizarin Red

BMD ..... Bone Mineral Density

BMI.....Body Mass Index

BMP..... Bone Morphogenic Protein

CBC .....Complete Blood Count

CKD.....Chronic Kidney Disease

CT .....Computed Tomography

CV..... Coefficient of Variability

DI ..... Deionized

DPBS .....Dulbecco's-Phosphate Buffered Saline

DXA ..... Dual Energy X-ray Absorptiometer

FBS .....Fetal Bovine Serum

FGF..... Fibroblast Growth Factor

FRAX .....Fracture Risk Assessment Tool

FSH.....Follicle Stimulating Hormone

GWAS .....Genome-wide Association Studies

LH..... Luteinizing Hormone

MSC.....Mesenchymal Stem Cell

OD .....Optical Density

pNPP.....P-Nitrophenol Phosphate

PTH..... Parathyroid Hormone

RANKL ..... Receptor Activator of Nuclear factor Kappa-B Ligand

SHBG .....Sex Hormone Binding Globulin

TGFB ..... Transforming Growth Factor-beta

Wnt ..... Wingless-type MMTV integration site

INTRODUCTION

As the most common bone disease in humans, osteoporosis is a significant public health problem which burdens the economy and the healthcare system. Osteoporosis is a multifactorial, polygenetic disease characterized by loss of bone quality that affects as many as 10 million Americans, both men and women of any age (Office of the Surgeon General, 2004). Many people do not know they have it until an osteoporosis-related fracture occurs (Sözen, Özışık, & Başaran, 2017). Approximately $17.9 billion per year is spent in the U.S. because of osteoporosis-related fractures, which have an estimated incidence of 1.5 million in the U.S. (Office of the Surgeon General, 2004). Although they account for less than 20% of all osteoporotic fractures, hip fractures have the worst outcomes and place the heaviest burden on health care resources (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy, 2001). It was estimated that more than 200 million patients worldwide would suffer from osteoporotic hip fractures by 2050, 6 times the fracture occurrence in 1992 (Cooper, Campion, & Melton, 1992).

Osteoporosis can affect anyone but has a higher incidence in Caucasians, women, and the elderly (Sözen, Özışık, & Başaran, 2017). Therefore, as the growing population ages, osteoporosis will become even more burdensome on society. Osteoporosis is defined as systemic bone loss and is accompanied by impaired bone mass, strength, and microarchitecture with increased risk for fragility fractures (Rachner, Khosla, & Hofbauer, 2011). Bone is continuously remodeled to repair minor damage and prevent major fractures, and when resorption outpaces formation, bone loss can occur. Bone mass

typically peaks around puberty and declines thereafter. The rate at which that decline occurs, and the subsequent risk of fracture, depends on many factors, including sex, general health, nutrition, endocrine influences, and physical activity. Genome Wide Association Studies GWAS)s have identified several loci associated with Bone Mineral Density (BMD) and osteoporosis. Bone mineral density is the amount of minerals, namely calcium and phosphorus, within a certain volume of bone. Most of these genes are members of the Wnt signaling pathway, the receptor activator of RANK/RANKL/OPG pathway, and developmental genes involved in endochondral ossification. Since BMD alone cannot account for the plethora of osteoporosis presentations, and not everyone with low BMD experience an osteoporotic fracture, GWASs are needed to help fill the gaps in understanding the processes underlying osteoporosis (Sabik & Farber, 2017).

Pathogenesis

Osteoporosis, which means "porous bone," is defined by the World Health Organization as a bone mineral density (BMD) T-score of 2.5 standard deviations or more below the mean BMD (World Health Organization, 1994 Mineralization stiffens the type I collagen scaffold and resists bending forces on the bone, but over-mineralization can result in excessive stiffness and subsequent brittleness. A balance between strength, lightness (porosity), and flexibility are needed for bone to appropriately respond to stress and dissipate force without damaging the bone (Seeman, 2002). The skeleton undergoes modeling throughout childhood and adolescence until epiphyseal closure near the end of puberty. This is followed by consolidation for 5 to 10 years until peak bone mass is

achieved, usually by the late 20s to early 30s (Lin et al., 2003). Bone is then constantly resorbed by osteoclasts and rebuilt by osteoblasts at an equal rate to maintain structural integrity. There are five stages of bone remodeling: the resting phase, activation phase, resorption phase, reversal phase, and formation phase. First, osteoclasts are recruited to the bone surface to create an acidic environment conducive to dissolving and resorbing the mineral content of the bone. Then, the osteoclasts undergo apoptosis and osteoblasts deposit collagen at the bone surface. The collagen is mineralized to form the new bone (Lewiecki, 2008).

Many factors are involved in bone remodeling regulation, including RANK, RANKL, and osteoprotegerin (Vega, Maalouf, & Sakhaee, 2007). RANKL is produced by osteoblasts and stromal cells and promotes osteoclastogenesis by binding RANK on osteoclast progenitor cells of the bone marrow. Osteoprotegerin is also made by osteoblasts and serves as a decoy to prevent RANK/RANKL binding. The amount of these molecules in the body is regulated by systemic hormones (i.e. estrogen), local factors (i.e. cytokines), and other factors. The mechanism for the regulation of site-specific remodeling is currently unknown (Watts et al., 2010). For various reasons, as a person ages, bone resorption gradually begins to exceed deposition so that there is a net loss of BMD (Rodan & Reszka, 2003). During stages of bone building, periosteal bone formation (outer surface) establishes the cross-sectional area of the bone, and endocortical bone formation (inner surface) determines cortical thickness. Endocortical resorption, called cortical thinning, decreases the distance between the endocortical and periosteal surfaces (Seeman, 2002). Bone marrow fat also accumulates with age as there

is a shift from osteoblastogenesis to adipogenesis (Pietschmann, Rauner, Sipos, & Kerschan-schindl, 2009). Both a low-turnover state, where bone resorption and formation are low, and a high-turnover rate, where resorption and formation are high, can favor resorption and result in osteoporosis (Boskey et al., 2005).

Other than BMD loss, changes in the microarchitecture of bone also contribute to the pathogenesis of osteoporosis. Microarchitecture includes trabecular thickness, connectivity, and porosity. Cancellous, or trabecular, bone is remodeled at 10 times the rate of cortical bone (Rodan & Reszka, 2003). When bone loss occurs, trabecular plates of the bone are lost, leaving the structure architecturally weaker and with less mass (Sözen, Özışık, & Başaran, 2017). In addition to overall trabecular bone loss, osteoporotic bone tends to show expansion of the periosteal envelope and cortical thinning that negatively affect bone quality (Seeman & Delmas, 2006).

Of all the factors that influence the ability of bone to withstand trauma - geometry, microarchitecture, stiffness, size - BMD accounts for the greatest variance in bone strength (Holroyd, Cooper, & Dennison, 2007). This is why osteoporosis, clinically defined as low BMD, is associated with such a high incidence of fractures (see Figure 1). Proximal femur fractures, or hip fractures, tend to have worse outcomes compared to vertebral fractures (Sözen, Özışık, & Başaran, 2017). They are associated with increased morbidity, disability, mortality, and overall decreased quality of life (Melton, 1997).

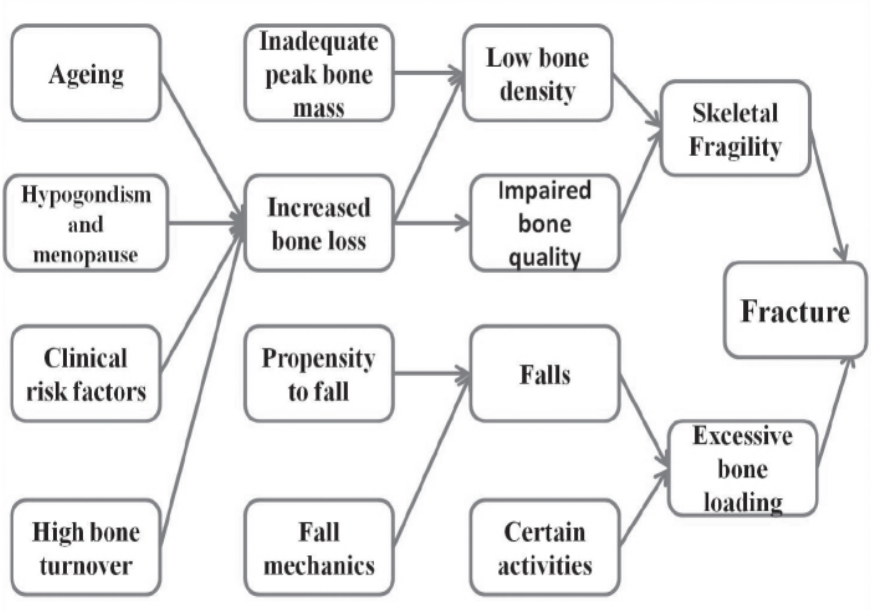

Figure 1. Pathogenesis of osteoporosis-related fractures. Adapted from "An overview and management of osteoporosis," by T. Sözen, L. Özışık, and N. Ç. Başaran. 2017, *European Journal of Rheumatology*, *4*(1), p. 48. Copyright 2017 by the Medical Research and Education Association.

Risk Factors

Despite the ongoing debate over the etiologies of osteoporosis, there is consensus that they all contribute to osteoporosis primarily by some mechanism that directly or indirectly affects bone turnover, microarchitecture, geometry, and BMD. As the clinical presentation of osteoporosis is most often following a fracture, conventional clinical risk factors focus more on fracture risk assessment instead of low BMD. As osteoporosis and fractures tend to go together, it is reasonable that the risk factors for developing osteoporosis are similar to the clinical risk factors in the Fracture Risk Assessment Tool Model (FRAX) used to assess the likelihood of a patient getting a fracture (i.e. age, sex,

low BMI (< 21kg/m^2), currently smoking, hyperthyroidism, frequent falling) (Sözen, Özışık, & Başaran, 2017). Osteoporosis can be divided into two groups, primary and secondary, depending on the causative factors affecting bone metabolism. Primary osteoporosis can be further subdivided into involutional osteoporosis type I, or postmenopausal osteoporosis, and involutional osteoporosis type II, or senile osteoporosis (Cosman et al., 2014).

Involutional osteoporosis type I. Postmenopausal osteoporosis (involutional type I) is a loss of BMD related to decreased estrogen production which affects 50% of post-menopausal women over 50 worldwide (Bidwell, Alvarez, Hood, & Childress, 2013). Estrogen deficiency is associated with osteoclasts and bone resorption, possibly by the use of cytokines (Rodan & Reszka, 2003). Estrogen deficiency affects estrogen receptors in osteoclast progenitor cells such that, as estrogen decreases, the inhibition on osteoclastogenesis is lifted and osteoclast activity increases. Certain cytokines that stimulate osteoclastogenesis (IL-1, IL-6, TNF) may also contribute to the pathology. Increased RANKL production in osteoblasts may also accelerate bone resorption, although the mechanism underlying estrogen stimulation on bone formation is less defined (Watts et al., 2010). Women undergo a period of four to eight years of trabecular bone loss by resorption related to estrogen deficiency around menopause. Another phase of bone loss, where both trabecular and cortical bone mass decreases, occurs later due to the effects of age on bone formation (Rogers et al., 2002).

Osteoporosis is four times more common in women than in men (Center et al., 1999). Sex steroid hormones play an important role in regulating bone mass in both

sexes, presumably estrogens in women and androgens in men. This may also account for some of the differences between osteoporosis rates and outcomes in men and women (Seeman, 2002). However, there is some support that estrogens are also important for male skeletal health. Estrogen and testosterone levels tend to be highly correlated, since most estradiol in men comes from peripheral aromatization of androgen precursors in circulation (Gennari, Khosla, & Bilezikian, 2009). Knockout mutations in the *CYP19* gene that encodes aromatase, which converts androgens to estrogens, results in estrogen deficiency and low BMD that often responds to estrogen therapy (Maffei et al., 2004). A large study by Mellström et al. (2008) analyzed testosterone and estradiol, their bioavailable fractions, and sex hormone binding globulin (SHBG) in elderly Swedish men. They found that testosterone and estradiol decreased with age while SHBG increased, and only free estradiol and SHBG were associated with fracture risk. Estradiol negatively correlated with fracture risk while SHBG positively correlated with it, but only estradiol was a significant predictor of fractures. Furthermore, researchers found that low estradiol correlated with increased fracture risk regardless of testosterone levels. If estradiol is indeed the primary sex steroid hormone defining osteoporosis and fracture risk in both sexes, then the question becomes about how a sudden drop in estradiol, such as with menopause, affects BMD differently than a gradual decline, such as is seen in men. Further research is needed to better understand this concept and other consequences of estrogen deficiency in both men and women. All things being equal, men and women appear to lose BMD differently; whereas postmenopausal women lose trabeculae (increase in trabecular separation), men undergo trabecular thinning without loss in

trabecular number (Aaron, Makins, & Sagreiya, 1987). Since trabecular number is more important for bone strength than thickness, this may also contribute to the sex difference (Silva & Gibson, 1997). Men also appear to have greater periosteal apposition, even under conditions of net bone loss, than women, which results in lesser overall bone loss and may offset some fracture risk (Seeman, 2002).

Other than differences in BMD and bone microarchitecture, there are several additional theories as to why the discrepancy in osteoporosis prevalence between sexes might be. Firstly, boys usually have higher bone density but take longer to develop it than girls do (Bonjour et al., 1991). Males also tend to have more lean mass than females, and lean mass has been associated with higher bone density in some studies (Nieves et al., 2005; Zhao et al., 2007). Women generally have smaller bones, which may be why women tend to start having fractures five to 10 years earlier than men and have a higher lifetime fracture risk (Wang, Duan, Beck, & Seeman, 2005). Moreover, women tend to start losing bone density at a younger age and faster rate than men (Jones et al., 1994; Hannan et al., 2000). Despite the increased incidence of osteoporosis and hip fractures in women, the mortality rate after hip fracture is greater in men (Haentjens et al., 2010).

Involutional osteoporosis type II. Senile osteoporosis (involutional type II) is a loss of BMD caused by processes related to aging. Age has numerous effects on BMD and therefore its relationship with osteoporosis is multifactorial. For example, when serum calcium is low, bone resorption is increased to raise it; since the elderly tend to have greater calcium needs, they are particularly susceptible to calcium deficiency (Sözen, Özışık, & Başaran, 2017). Furthermore, mesenchymal stromal cells give rise to

both osteoblasts and adipocytes. Aging may shift differentiation and subsequently affect bone marrow composition by increasing adipocytes and decreasing osteoblasts, which could result in decreased bone mass and osteoporosis (Horowitz & Lorenzo, 2004). Vitamin D, which is necessary for calcium absorption, can be deficient in older patients, particularly if they are obese, have dark skin, limited sun exposure, have various gastrointestinal disorders, or are on certain medications (i.e. certain anticonvulsive drugs increase vitamin D breakdown). There is also the simple fact that osteoporosis is usually detected after a related fracture. Fractures often accompany falls, and the elderly are particularly susceptible to falling (Sözen, Özışık, & Başaran, 2017). This risk can be compounded by frailty, sarcopenia, and other common comorbidities such as dementia (Aspray & Hill, 2019). Alzheimer's disease, another chronic degenerative disorder primarily found in the elderly, is associated with an increased incidence of hip fractures and increased mortality within one year of fracture (Baker, Cook, Arrighi, & Bullock, 2011). Alzheimer's shares many of the same risk factors as osteoporosis (i.e. lower body mass, vitamin D deficiency, decreased sunlight exposure, decreased physical activity) and may predispose elderly persons to osteoporosis (Tysiewicz-Dudek, Pietraszkiewicz, & Drozdzowska, 2007). Another theory is that amyloid beta (AB) has neurotoxic effects, specifically it increases levels of H_2O_2 which induces osteoclastogenesis (Jules et al., 2012). All of these are potential mechanisms for how ageing increases the risk for osteoporosis and related fractures.

Secondary osteoporosis. Secondary osteoporosis is associated with many different medications, diseases, and lifestyle factors (Cosman et al., 2014). Table 1 gives

a brief overview of secondary variables associated with osteoporosis (Cosman et al., 2014; table adapted from Sözen, Özışık, & Başaran).

Table 1

Causes of Secondary Osteoporosis

Lifestyle factors	Genetic Diseases	Endocrine Disorders	Other
Vitamin D sufficiency	Glycogen storage diseases	Central obesity	Congestive heart failure
Smoking (active or passive)	Osteogenesis imperfecta	Cushing's syndrome	End stage renal disease
Alcohol abuse	Parental history of hip fracture	Diabetes mellitus (1 and 2)	Hypercalciuria
Inadequate physical activity	Ehler Danlos	Androgen insensitivity	AIDS/HIV
Excessive thinness	Hemochromatosis	Anorexia nervosa	Weight loss
Low calcium intake	Homocystinuria	Premature menopause (<40 years)	Post-transplant bone disease

Lower body weight (BMI $\leq 19 kg/m^2$) has been associated with lower BMD, increased bone fragility, and increased incidence of fracture (Frost, 1995) The relationship between obesity and osteoporosis is more complicated and, therefore, more controversial. Obesity, generally defined as a BMI of 30 or over, is widely believed to be associated with increased bone mass. This is because the larger body mass generates a greater load on the skeleton, and the skeleton increases in mass to accommodate. Obesity

may be primarily protective against osteoporosis in older women since adipocytes are a source of estrogen in postmenopausal women (Kameda et al., 1997). However, one study found that, when the mechanical loading effect of body weight is statistically removed, obesity (measured by body fat mass and not BMI) was negatively correlated with bone mass. In other words, individuals with more body fat had less bone mass. They also found a positive correlation between lean mass and bone mass regardless of body weight (Zhao et al., 2007). These findings support the notion that a greater load on bone increases bone mass, but challenges the idea that fat is protective against bone loss and therefore, osteoporosis. Other studies suggest that hormones like leptin, adiponectin, and adipsin, which are all secreted by adipocytes, contribute to the pathogenesis of osteoporosis (Sharma, Tandon, Mahajan, Mahajan, & Mahajan, 2014). Again, this opposes the idea that fat is protective against osteoporosis. Further research, including large population-based studies, are needed to better understand the effects of body fat and weight on osteoporosis risk.

Chronic kidney disease (CKD) has been associated with a 4.4 times greater risk of fracture than the average population, and in women a 16% increase of hip fracture for every 1-standard deviation elevation of serum cystatin C (Alem et al., 2000; Fried et al., 2007). This may be due to secondary hyperparathyroidism, adynamic bone, hemodialysis-associated amyloidosis, vitamin D deficiency, hypocalcemia, bone architecture changes, nutritional differences, or increased oxidative stress (Nitta, Yajima, & Tsuchiya, 2017). When CKD advances beyond stage 3, urinary phosphorus excretion decreases to such a point that fibroblast growth factor 23 (FGF23) secretion from bones

(which normally increases urinary phosphorus excretion) cannot compensate. The elevation of FGF23, when accompanying minor calcium deficiency, leads to decreased vitamin D activation and enhanced PTH secretion. This can result in secondary hyperparathyroidism due to phosphorous loading, which can prompt fibrous osteopathy and high rates of bone turnover (Salam, Eastell, & Khwaja, 2014). Therefore, CKD-associated osteoporosis and the increased bone fracture incidence may be due to phosphorus accumulation in plasma (Chen & Moe, 2015). The effects of renal disease on osteoporosis may be so significant that BMD is not a reliable risk factor in these patients, however additional research is needed to fully understand this relationship (Dukas, Schacht, & Stähelin, 2005).

Smoking cigarettes is related to lower bone mass and an increased risk of fractures. This may be because serum parathyroid hormone, along with vitamin D, plays a significant role in calcium homeostasis and bone metabolism, and serum PTH has shown to be elevated in heavy smokers (Ortego-Centeno et al., 1997). Additionally, smoking is associated with decreased levels of 1,25-dihydroxyvitamin D. Theoretically, the combined effects of decreased vitamin D and PTH is that osteolysis increases and bone formation decreases (Brot, Jorgensen, & Sorensen, 1999). In pre-menopausal women, smoking is associated with increased FSH and LH, which decreases estrogen levels (Kline, Tang, & Levin, 2016). The consequences are similar to that in postmenopausal women: bone loss and increased incidence of osteoporosis. Although there is research to support that heavy smoking increases osteoporosis risk, there is conflicting evidence for the consequences of passive smoking and debate over what the

underlying mechanism is between smoking and bone loss (Pouresmaeili, Kamalidehghan, Kamarehei, & Goh, 2018). In experiments using mouse cell lines as osteoblasts and osteoclasts that were treated to 4% cigarette smoke over 14 weeks, researchers found that the smoking group had significantly higher urinary ALP and mineral levels, indicated increased bone metabolism. The smoking group also exhibited femoral osteopenia by decreased bone volume and trabecular thickness. Isolated cell studies demonstrated that smoking inhibited osteoblast differentiation and subsequent bone formation, but it promoted osteoclast differentiation (Ko et al., 2015). These results support the idea that passive smoking can have severe consequences on bone cell differentiation and bone remodeling, however human studies have yet to support this notion.

Research has supported that there are race differences in the prevalence and outcomes of osteoporosis. The National Health and Nutrition Examination Survey covering 2005 to 2006 found that 10% of white women had osteoporosis compared to 6% of African Americans, and 10% of Hispanics. Although the incidences for osteoporosis were similar, the prevalence of low bone mass declined between whites (50%) and Hispanics and African Americans (38% and 39%) (Looker et al., 2010). In the United States, annual hip fracture rates are highest in white women (140.7 per 100,000), then Asian women (85.4 per 100,000, African American women (57.3 per 100,000), and Hispanic women (49.7 per 100,000) (Silverman & Madison, 1988). Research regarding fracture rates in men are a little more contradictory. Some studies have found that, although hip fracture rates were slightly greater for white men than other minorities, the difference wasn't as significant as for women (Cummings & Melton, 2002). Another

study found the risk difference to be as much as 50% between older Asian men and Caucasian men (Lei et al., 2006). There is clearly an interplay between sex and ethnicity/race on osteoporosis and fracture risk, and additional research is needed to fully understand those relationships. The differences seen in osteoporosis rates across races may be due to different prevalence of risk factors. For example, African American women tend to have higher BMD than white women regardless of body weight (Finkelstein et al., 2008). Other differences may include differences in hip geometry, lifestyle factors, and prevalence of other comorbidities (Cauley, 2011).

Prevention and Treatment

It is generally recommended that all at-risk patients receive an adequate intake of calcium and vitamin D as part of osteoporosis prevention. Additionally, increasing exercise, decreasing alcohol intake, and discontinuing tobacco use are recommended (Sözen, Özışık, & Başaran, 2017). The most pertinent sequelae of osteoporosis are fractures and their associated consequences, including chronic pain, disability, and death. Therefore, a primary goal of osteoporosis treatment is prevention of fractures and symptom relief. Proximal femur fractures in particular are associated with a 15-20% increased mortality rate within one year (more in men than women), a 2.5 times greater chance of future fractures, and frequently require long-term nursing home or other inpatient facility care for rehabilitation (Melton, Achenbach, Atkinson, Therneau, & Amin, 2013). Since fractures usually occur after some sort of fall, prevention programs aim at reducing fall risk in the elderly, confused, weak, and frail persons. Facilities that cater to these persons, such as nursing homes and long-term acute care facilities, often do

rehabilitation involving weight-bearing exercises to strengthen the legs and back in addition to balance training. Padding, particularly on the hips, has been shown to reduce the risk of fracture even if it doesn't reduce the risk of the fall itself (Sözen, Özışık, & Başaran, 2017). Education and awareness of osteoporosis and its risk factors are also important steps in any prevention program (Burke-Doe et al., 2008).

Diagnosis is made from patient history, occurrence of a fragility fracture (no major trauma), imaging studies, and measurement of the bone mineral density (Sözen, Özışık, & Başaran, 2017). Bone histomorphometry is the most accurate test for osteoporosis, but it is invasive. Therefore, BMD may be used to estimate bone mineral content, which has shown to correlate with bone strength in vitro. This is done by taking an x-ray proximal to a common osteoporotic fracture site (hip, spin, or wrist) and measuring the x-ray beam attenuation using dual energy x-ray absorptiometer (DXA) calibrated against a bone/soft tissue "phantom" (Rudang et al., 2016). As research evolves, new methods are developed to better diagnose and understand osteoporosis. This includes the use of high-resolution CT to determine bone microarchitecture (trabeculation and porosity) and biochemical tests of bone formation and resorption (Aspray & Hill, 2019). Adults 50 years and older considered "high-risk" for fracture (as assessed by FRAX) or with present fractures of the vertebrae, proximal femur, distal forearm, or shoulder should be assessed for osteoporosis, however lack of cohesion in fracture assessment worldwide has led to wide variation in diagnosis and treatment (Cosman & Melton, 1992; Curtis et al., 2017; Sözen, Özışık, & Başaran, 2017). Secondary causes of osteoporosis can often be inferred from a thorough medical history and physical

examination (Sözen, Özışık, & Başaran, 2017). Additional testing, including CBC, serum calcium, alkaline phosphatase (ALP), and vitamin D, can rule out secondary osteoporosis (Tannenbaum et al., 2002).

In additional to surgical interventions (fracture repair and joint replacement), there are several pharmacological treatments for osteoporosis. These include, but aren't limited to, calcium and vitamin D supplementation, hormonal replacement therapy, and bisphosphonates (Hernlund et al., 2013). Antiresorptive agents such as estrogen, bisphosphonates, selective estrogen receptor modulators, human monoclonal antibody against RANKL, and strontium ranelate, can be prescribed for the prevention of osteoporosis and related fractures (Sözen, Özışık, & Başaran, 2017). Denosumab, a human monoclonal antibody that targets RANKL, was shown to reduce vertebral fractures and hip fractures by 68% and 40%, respectively (Cummings et al., 2009). It should be noted that, although antiresorptive agents increase bone mass, they do not stimulate bone formation and not all of them are associated with decreased fractures (Sözen, Özışık, & Başaran, 2017). Despite the development of newer and more effective treatments for osteoporosis, there is evidence to suggest that only a minority of people suffering from osteoporosis get treatment. This treatment gap poses both a person burden on the patient and a societal burden on the healthcare system, since the risk of additional fractures is higher in untreated patients (Hernlund et al., 2013). Guidelines addressing the discord in osteoporosis risk assessment and diagnosis may also help alleviate the osteoporosis treatment gap and, therefore, the burdens that come with it (Clynes et al., 2020).

Osteogenesis

Osteoblasts differentiate from multipotent mesenchymal stem cells (MSCs) (see Figure 2). Then they become inactive lining cells, osteocytes, or undergo apoptosis. Osteoclasts, however, are derived from hematopoietic stem cells (Pietschmann, Rauner, Sipos, & Kerschan-schindl, 2009). MSCs are easily isolated and expandable, making them useful in both clinical and research settings. Bone marrow-MSCs, as the name implies, are typically isolated from whole bone marrow aspiration and easily adhere to plastic cell culture plates, making them one of the best stem cell sources (Tondreau et al., 2004). The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed that, to qualify as human MSCs, the cells must be plastic-adherent; express CD105, CD73, CD90 and lack expression of CD45, CD34, CD11b, CD79a, CD19, and HLA-DR surface molecules; and they should be able to differentiate into osteoblasts or adipocytes in vitro (Pittenger et al., 1999). Differentiation of MSCs involves lineage commitment and maturation from progenitors to the specific cell types. Several signaling pathways are involves in lineage commitment of MSCs, including TGF*B*/BMP signaling, Wnt signaling, Hedgehogs, Notch, FGFs, and more. MicroRNAs, such as miR-204 and miR-335, and various transcription factors have also been implicated in lineage commitment of MSC. Factors affecting the balance between adipogenic and osteogenic differentiation of MSCs may be implicated in the pathogenesis of osteoporosis and could be targeted by future treatments. In vitro differentiation of MSCs into osteoblasts is well established and can be done chemically using osteogenic medium (Chen et al., 2016). MSC differentiation ex vivo by use of osteogenic medium

encourages mineral formation in culture. Previous studies have found the mineralized nodules were positive for Alizarin Red staining (Pittenger et al., 1999). Alizarin Red S (ARS) staining facilitates estimation of mineral deposition and inspection of fine structures when used with phase contrast microscopy. ARS dye can be extracted from the culture and assayed for more precise quantification (Gregory, Gunn, Peister, & Prockop, 2004).

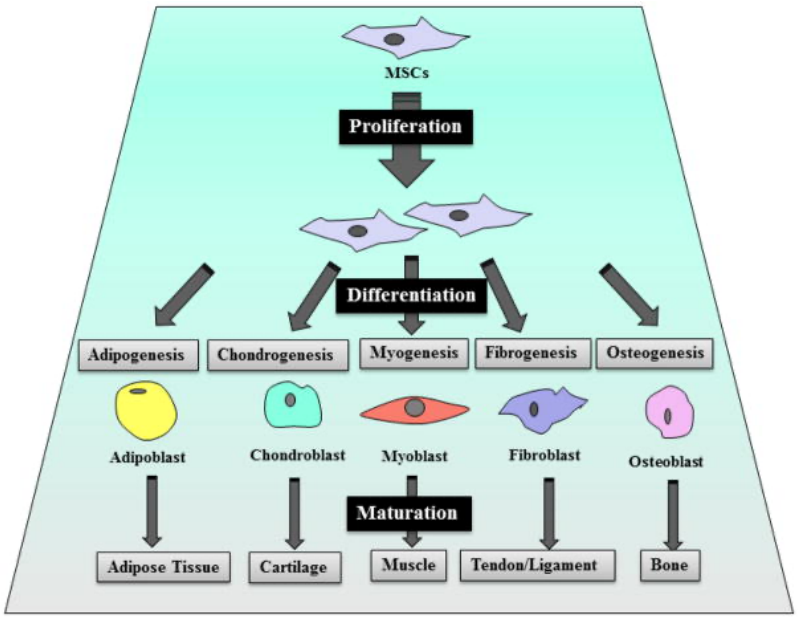

Figure 2. Pathways for bone marrow mesenchymal stem cell differentiation. Adapted from "Key transcription factors in the differentiation of mesenchymal stem cells," by S. G. and D. K. Agrawal, 2016, *Differentiation, 92*(1-2), p. 41–51. Copyright 2020 by Elsevier B. V. Reprinted with permission

Although BMD can be measured, the only clinical indicators for the degree of the biological processes that effect bone tissue deterioration, and therefore rapid bone remodeling, are the biochemical markers (Seeman & Delmas, 2006). These include resorption markers (serum C-terminal telopeptide type-I collagen and urinary N-telopeptide) and formation markers (serum procollagen type-I N-terminal pro-peptide)

(Sözen, Özışık, & Başaran, 2017). These markers are indicative of bone remodeling at any one time and can be used clinically to determine the effectiveness of treatments (Unnanuntana, Gladnick, Donnelly, & Lane, 2010). Another method to determine the rates of bone formation and degradation is to measure the enzymatic activity of osteoblastic or osteoclastic cells or to measure components of the bone matrix released during formation or resorption.

Alkaline phosphatase (ALP) is an enzyme found in abundance throughout the body, in bone and other tissues. Bone-specific ALP is a bone formation marker. When bone forms, osteoblasts produce type-I collagen and various non-collagenous proteins, such as ALP and osteocalcin (Unnanuntana, Gladnick, Donnelly, & Lane, 2010). Osteogenic induction of MSCs initiates mineral aggregation and increases alkaline phosphatase activity (Pittenger et al., 1999).

In endochondral ossification, the mechanism of bone development from a cartilaginous bone model that is used by most higher vertebrates, calcification occurs at matrix vesicles in the lacunae. These nucleation sites accumulate calcium and phosphate, which serve to help form the main inorganic component of bone, hydroxyapatite (Gregory, Gunn, Peister, & Prockop, 2004). Hydroxyproline is an amino acid found primarily in collagen, and since it is not reusable, it can be used as an indicator of collagen breakdown. Since half of human collagen resides in bone, and bone turnover is faster than other collagenous soft tissues, hydroxyproline is considered a marker of bone resorption. Research has indicated that, when serum ALP is elevated, so is urinary hydroxyproline excretion in patients with various bone diseases (Cerda, Toskes, Shopa,

& Wilkinson, 1970). This indicates that the two enzymes together, a formation marker and a resorption marker, may be indicative of increased bone remodeling rates.

Present Study

The present study investigates the relationship between various osteoporosis risk factors and biochemical markers of osteogenic bone cell growth and metabolic activity, and the reproducibility of these assays. The risk factors include sex, age, race, BMI, vitamin D level, renal disease status, and smoker status. The goal of the study is to correlate the biochemical markers and identify significant relationships between the biochemical markers and risk factors. The first hypothesis is that patients with greater MSC growth and differentiation will show increased ARS scores, ALP activity, and hydroxyproline. It is expected that the assays will show a positive correlation between each other as they are different methods to quantify differentiation. Research has established that the highest rates of osteoporosis are seen in postmenopausal white women, possibly due to a decline in systemic estrogen and an increase in osteoclast activity. Therefore, the second hypothesis of this study is that white women over 50 years of age will have the greatest decrease in concentrations of ALP with increased hydroxyproline. Insights gained in this study will help determine the relationship between various factors and osteoporosis as measured by markers of bone resorption and deposition and guide further analyses in the larger study that aims to identify the genes underlying osteoporosis.

METHODS

Participants

Participants (N = 26, 15 males and 11 females) in this study were all patients that underwent total hip arthroplasty surgery at Boston Medical Center (BMC) between 2019 and 2020. The average age was 53.12 years old (SD = 9.43) and ranged from 36 to 73 years old. For analyses, patients were grouped into two categories: those under 50 years old (N = 8, 30.8%) and those who were 50 years old and over (N = 18, 69.2%). Study subjects self-identified as white (N = 11, 42.3%) African American (N = 12, 46.2%), Asian (N = 1, 3.8%), other (N = 2, 7.7%), or declined to answer (N = 2, 7.7%). Two subjects (7.7%) identified as Hispanic or Latino. The Body Mass Index (BMI) of patients ranged from 21.16kg/m^2 to 41.39kg/m^2 (M = 30.29, SD = 5.22). For the purpose of this study, overweight was defined as a BMI greater than or equal to 30kg/m^2. Using this definition, 12 patients were categorized as healthy (46.2%) and 14 were identified as overweight (53.8%). Patients were also grouped based on their vitamin D status, with low being less than 30ng/mL (N = 16, 61.5%) and high being 30ng/mL or greater (N = 7, 30.4%). Three patients were excluded because their vitamin D levels weren't available. Four patients had some form of diagnosed renal disease (15.4%), and 22 patients did not (84.6%). For smoking status, six patients self-identified as smokers (23.1%), eight identified as non-smokers (30.8%), and 12 identified as former smokers (46.2%).

Patients were excluded from the study if they had certain comorbidities such as sickle cell, rheumatoid arthritis, HIV, or Hepatitis C; if they were receiving chemotherapy; or if they had taken drugs or had other treatments that alter bone

metabolism or modify bone in any other way. All human research was done under a Boston University School of Medicine Institutional Research Board Approved protocol: "Bone Tissues Repository", IRB Number: H-35199. Graduate students consented the patients in accordance with current HIPAA regulations prior to surgery and specimen collection.

Cell Culture Procedure

Isolating MSCs from reamings. The femoral head and reamings, from the coring of the acetabulum, were collected fresh during total hip arthroplasty. The contents were placed in sterile capped specimen cups and transported back to the Orthopedics Laboratory at Boston University School of Medicine on ice in a biohazard cooler. The weight of the total bone marrow contents was obtained by calculating the difference between the weight of the specimen cup with the sample and without the sample. All work with the marrow cell products was carried out in a certified biocontainment tissue culture hood. The bone marrow was transferred into a sterile 400mL Pyrex wide mouth jar with 100mL of DPBS (Hyclone Laboratories, Inc., Logan, UT) and 10mL of 100X antibiotic-antimycotic (Thermo Fischer Scientific, Waltham, MA). The jar was sealed and shaken vigorously for 30 seconds. The cell-containing wash was poured through a sterile stainless-steel cell sieve to another sterile 500mL Pyrex jar retaining the larger tissue reamings and marrow tissues in the original jar. Another 100mL of DPBS and 10mL of anti-anti were added to the original jar with the marrow. The contents of the jar were again sealed, shaken, and passed through the sieve into the second jar. The strained

wash material containing soluble cells and small tissue fragments was used for further processing.

MSC processing. A 120mL aliquot of the wash was divided into 40mL aliquots in each of three 50mL falcon tubes (Corning Inc., Corning, NY). The tubes were centrifuged in a Centrifuge 5810 R Eppendorf Desk Top centrifuge at 1150rpm using a 4x250mL swinging bucket rotor for five minutes. The top 5mL of wash containing a distinct fat layer was aspirated off of each falcon tube to reduce the number of filtrations required in the following step (see Figure 3). For each tube of sample, the wash was shaken vigorously to resuspend the pellet and subsequently passed through a 70µm then a 40µm cell strainer (Corning Inc., Corning, NY) into new falcon tubes (see Figure 4). Cells were concentrated by centrifugation at 1150rpm for five minutes three subsequent times, suspending the packed cells sequentially in 35mL of DPBS, then two additional times in 10mLs. After the final wash, the packed cells were pooled into a single tube and suspended in a final 10mL DPBS.

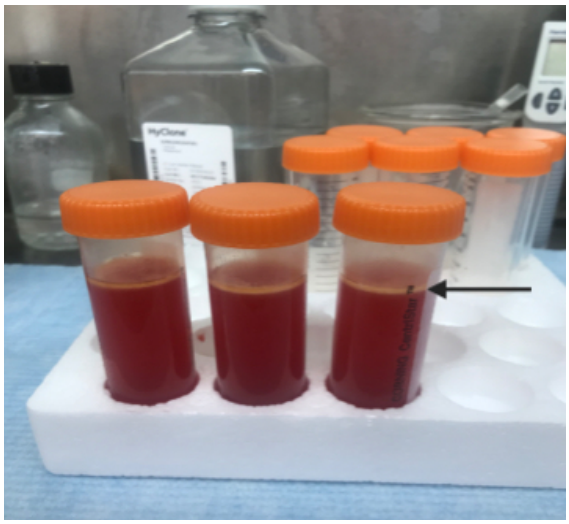

Figure 3. Fat layer on top of marrow wash. Created with BioRender.com.

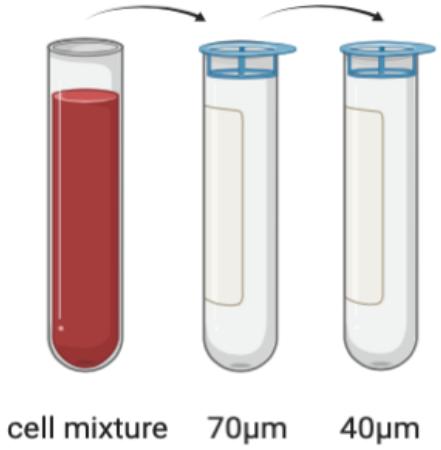

Figure 4. Cell straining procedure. Created with BioRender.com.

MSC plating. The cells were then manually counted using a hemocytometer and a 1:100 cell wash dilution made by suspending 10µL of the cell stock into 990µL DPBS. The cells were seeded at $2.66x10^6$ cells/cm^2: 24 million cells/well for the 6-well plates, and 5.32 million cells/well for the 24-well plates.

Prior to cell seeding, the 6-well and 24-well tissue-culture plates (Corning Inc., Corning, NY) were treated with Animal Component-Free Cell Attachment Substrate (Stem Cell Technologies, Vancouver, Canada) diluted 1:150 in DPBS (i.e. 100µL attachment substrate in 15mL DPBS). The substrate was gently mixed by inversion and used to coat the culture ware using 250µL/well for 24-well plates and 1mL/well for 6-well plates). The plates were tilted to ensure even coating of the solution over the surface and incubated for at least two hours at room temperature. The substrate solution was aspirated off, using caution to avoid scratching the surface, and the culture ware was washed once with DPBS at 0.5mL/well for the 24-well and 2mL/well for the 6-well. Once the wash was carefully aspirated off, the culture ware was ready for plating.

Each cell suspension in basal media was plated on the culture ware, respectively. For the 6-well plate, 2mL of cells were plated per well. For the 24-well plate, 0.5mL of cells were plated per well. The plates were grown in an incubator at 37˚C, 5% CO_2, and >90% humidity.

MSC growth. A half media change was performed on day four after MSC plating by removing half the volume of media and replacing it with the same volume of fresh basal media (1mL per well for 6-well and 250µL per well for 24-well). This was done in a way that minimized disruption of the cells.

A full media change was performed on day six after MSC plating by removing all the media and replacing with the same volume (2mL per well for 6-well and 0.5mL per well for 24-well) osteoinductive media. Osteoinductive media was introduced to the cells on day six. The osteoinductive media was made up of 200mL Osteogenic Differentiation Basal Medium (Stem Cell Technologies #05466), 50mL 5X Supplement (Stem Cell Technologies #05467), 2.5mL 200mM L-Glutamine (2mM final concentration), and 2.5mL 100X antibiotic-antimycotic (anti-anti). The plates were grown for 21 days in osteoinductive media and underwent a media change 3 times a week.

MSC harvest. Cultures were harvested 21 days after osteoinduction, bringing the total plate incubation time to 27 days. One six-well and one 24-well culture was carried for assaying each patient. The 24-well plates were used for sequential assays for alkaline phosphatase (ALP), DNA, total protein, total hydroxyproline, and total calcium. The alizarin red (ARS) assay was carried out on three of the six wells of each six-well dish, while the other three wells were retained for RNA purification. The ALP and ARS assays

were performed concurrently during harvesting. The media was aspirated off the plates, and they were washed with 0.5mL per well of DPBS. Next, the ALP assay and ARS quantification were performed (see protocol below). After completion of the ALP assay, the 24-well plate wells were washed with 0.5mL per well of DPBs to remove any residual pNPP solution, and the cells were harvested for the sequential biochemical assays. For harvesting, 100µL of extraction buffer (4M Guanidine-HCl, 1% Triton X-100, 10mM Tris HCl, 2mM EDTA (pH 7.4)) was added to each well, and the plate was placed on a shaker for 30 minutes. The wells were scraped to detach the cells, and each well-s contents were transferred to separate microcentrifuge tubes with 100µL of ultrapure water. The tubes were labelled to preserve well-matching and stored at -80˚C until assayed.

Alkaline Phosphatase Assay. To each sample well, 450µL of ALP assay buffer and 50µL of ALP substrate were added. The 1x ALP assay buffer consisted of 0.75g of 0.1M glycine per 100mL of DI water and 9.5mg of 1mM MgCL2 per 100m DI water. The pH was adjusted to 10.5, filtered, and stored at 4˚C for up to 6 months. The ALP substrate was made by dissolving 20mg of p-nitrophenol phosphate disodium salt (pNPP; Sigma #4876-1bm) per 1mL of DI water. The substrate was made on the day of assay and covered with foil until use as it is light-sensitive. After adding the buffer and substrate, the plate was incubated in the dark for 30 minutes at room temperature. A new, clear 24-well plate was prepared with 0.5mL per well of 0.2N NaOH. After 30 minutes, the pNPP solution was transferred to the second plate containing NaOH to neutralize the reaction and the absorbance read at 410nm. The plate was washed with 0.5ml/For the standard,

10mM p-nitrophenol stock solution (Sigma N7660-100mL) diluted to make a 1mM stock (100µL 10mM P-nitrophenol in 900µL of DI water. The 1mM p-nitrophenol was diluted with ALP buffer and 0.2N NaOH to make a standard curve (see Table 2).

Table 2

Preparation of ALP Standard

Volume p-nitrophenol stock (µL)	Volume ALP buffer (µL)	Volume 0.2N NaOH (µL)	Final Concentration (nM/mL)
5	495	500	5
10	490	500	10
20	480	500	20
30	470	500	30
40	460	500	40
50	450	500	50
60	440	500	60
80	420	500	80
100	400	500	100
120	380	500	120
140	360	500	140
160	340	500	160

The standard was prepared on a separate 24-well plate and the absorbance read at 410nm (see Figure 5).

5nM/ml	10nM/ml	20nM/ml	30nM/ml	40nM/ml	50nM/ml
60nM/ml	80nM/ml	100nM/ml	120nM/ml	140nM/ml	160nM/ml
Blank	Blank	Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank	Blank	Blank

Figure 5. ALP standard plate layout

Alizarin Red S Quantification Assay. The 6-well plate was used for ARS quantification. The plate was first washed several times with double distilled water then three of the wells were stained with 2% alizarin red solution for 30 minutes. The alizarin red solution consisted of 2g alizarin red S (Acros Organics, Fisher) per 100mL of deionized water. The solution pH was adjusted to 4 using 1% ammonium hydroxide solution and passed through a sterile filter. The solution was stored at 4˚C and made fresh every two weeks. After 30 minutes, the plate was rinsed with double distilled water. Each of the three stained wells were visually inspected and categorically ranked by nodule number (1 = low, 2 = moderate, 3 = high). The wells were averaged and each patient assigned a single whole integer classification indicative of nodule presence (see Figure 6, Figure 7, Figure 8.

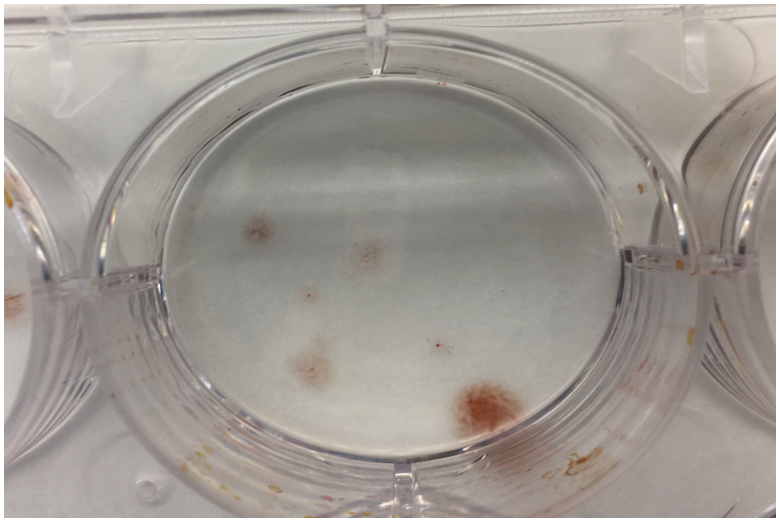

Figure 6. Example of alizarin red s low (1) nodule classification

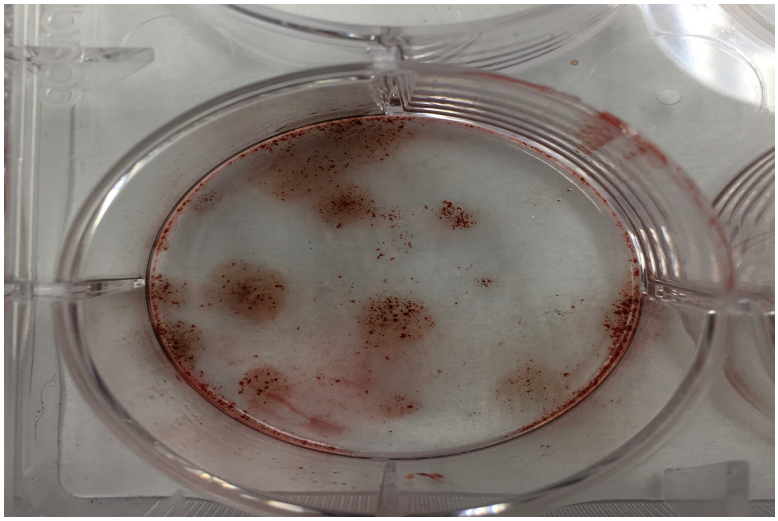

Figure 7. Example of alizarin red s moderate (2) nodule classification

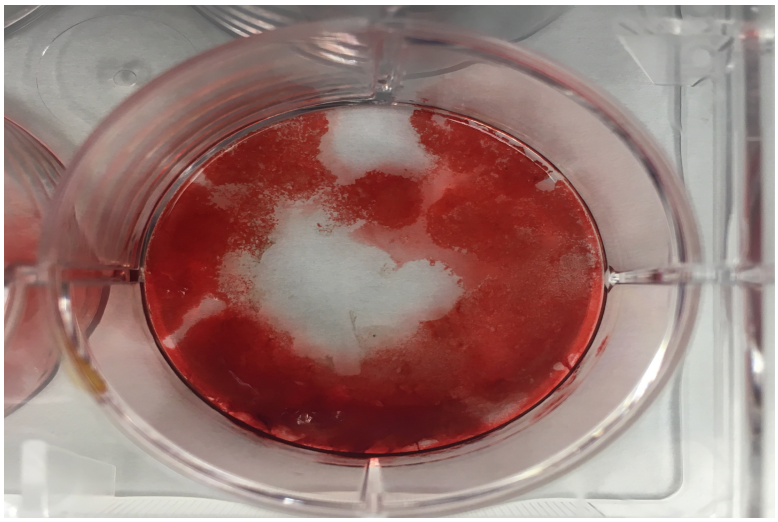

Figure 8. Example of alizarin red s high (3) nodule classification

Assay Protocol

On the day of assay, the microcentrifuge tubes were thawed on ice and spun down at 12000rpm for 5 minutes at 4˚C. The microcentrifuge tubes were labelled so that each assay well matched the incubation plate well for each patient (A1 was always A1, A2 was always A2, etc.). Each well on a harvest dish corresponded to one well on each assay; samples were not run in duplicate. Aliquots of 25µL were taken from each tube for the DNA and protein assay (50µL per tube in total). An acid hydrolysis was performed before the calcium and hydroxyproline assays were run. Two different methods of hydrolysis were used throughout the study in an attempt to correct errors with the calcium assay. For the first method, 150µL of concentrated HCl was added to each microcentrifuge tube and hydrolyzed at 120˚C for 3 hours. The tubes were then cooled to room temperature and spun down at 12000rpm at 18˚C for five minutes to remove any particulate. Aliquots of 100µL and 200µL were taken from each tube for the calcium and

hydroxyproline assays, respectively, and both plates were dried in a vacuum oven at 60˚C for 24 hours (or until all the acid evaporated) before analysis. For the second method, 13.0µL of 12N HCl was added to each microcentrifuge tube. The tubes were then vortexed and allowed to extract for 30 minutes at room temperature. For the calcium assay, 55µL were removed from each tube and plated. For hydrolysis, an additional 92µL of 12N HCL was added to each tube before they were placed in a heating block in the fume hood and incubated for three hours at 120˚C. The tubes were cooled to room temperature, spun down, and 200µL of each sample were plated for the hydroxyproline assay. The plate was incubated at 60˚C for about 24 hours to evaporate all the acid before being assayed. All of the assays were performed using commercially available kits, which were stored at 4˚C until used for analysis. The protein assay kit was able to be stored at room temperature. Optical density for the colorimetric assays was determined using a BioTek Synergy 2 Multi-mode Microplate Reader (BioTek Instruments Inc., Winooski, VT).

DNA assay. DNA was assayed first to minimize the effect of DNAses. The DNA assays were performed on black 96-well microplates using commercially available Thermo Fisher Scientific PicoGreen dsDNA reagent kits. The standard was made by serial dilution of the stock standard (see Table 3).

Table 3

Preparation of DNA Standard

Vial	1X TE (µL)	DNA (µL)	Final Concentration (ng/mL)
A	1000	10 Stock	1000
B	500	500 Vial A	500
C	200	400 Vial B	200
D	100	400 Vial C	100
E	50	250 Vial D	50
F	5	50 Vial E	5

A standard curve was included on each plate by adding 100µL of each standard dilution to separate wells on the plate (see Table 2). After adding 25µL of soluble sample extract from each microcentrifuge tube to the plate, 75µL of 1x TE was added to each well to bring the total sample volume per well to 100µL. The Picogreen reagent was diluted 1:200 in 1x TE, and 100µL was added to each sample and standard well on the plate. The plate was then covered with foil (Picogreen is light sensitive) and placed on a shaker for 5 minutes before being read on a fluorescent plate reader at excitation/emission: 485/20nm and 530/25nm, optics position top 50%, sensitivity 50, and height 7mm (see Figure 9).

Figure 9. DNA assay with two patients' samples and standard

Protein assay. The protein assays were performed on clear 96-well plates using commercially available Thermo Fisher Scientific Micro BCA Protein Assay kits. The standard was made by serial dilution of one 1.0mg/mL albumin (BSA) standard ampule (see Table 4).

Table 4

Preparation of Diluted Albumin (BSA) Standard

Vial	Volume TE (µL)	Volume/Source of BSA (µL)	Final [BSA] (µg/mL)
A	900	100 Stock	200
B	100	300 Vial A	150
C	200	200 Vial A	100
D	200	100 Vial B	50
E	600	100 Vial A	40
F	300	300 Vial E	20
G	300	300 Vial F	10
H	300	300 Vial G	5
I	300	300 Vial H	2.5
J	300	200 Vial I	1
K	200	200 Vial J	0.5
L	400	N/A	Blank

A standard curve was included on each plate by adding 25µL of each standard to separate wells on the plate. Each sample well got 125µL of 1xTE, and each standard well got 100µL of 1xTE. An additional 25µL of extraction buffer was added to each standard well. The working reagent (WR) was prepared at a 25:24:1 ratio of reagents MA:MB:MC (provided by assay kit). Each sample and standard well had 150µL of WR added to it. Then, the plate was mixed on a plate shaker for 30 seconds, covered with sealing tape, and incubated at 37˚C for two hours. The plate was cooled to room temperature and the absorbance read at 562nm (see Figure 10).

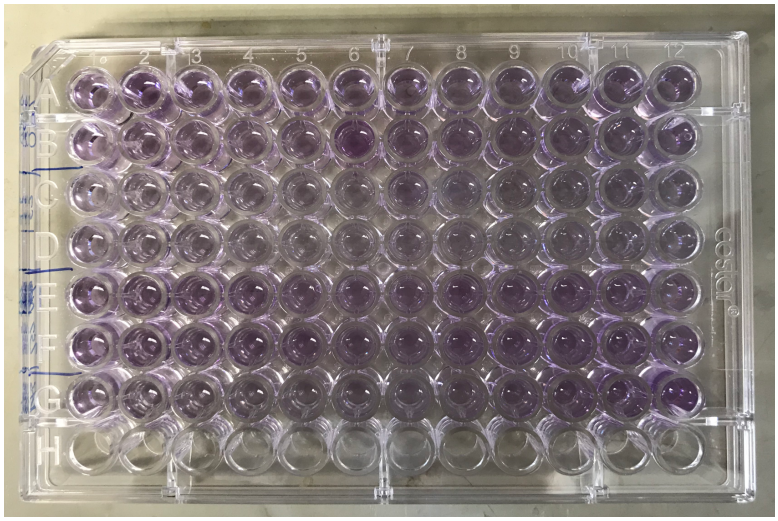

Figure 10. Protein assay with three patients' samples and standard

Calcium assay. The calcium assays were performed on clear 96-well plates following acid hydrolysis using commercially available Sigma-Aldrich Calcium Colorimetric Assay kits. The protocol for calcium was changed partly through as new research revealed that a different approach may be better. For both protocols, 10µL of the 500nM standard stock were diluted with 990µL of ultrapure water for a final concentration of 5nM. A standard curve was made by adding 50µL of each standard dilution to separate wells on the plate (see Table 5).

Table 5

Preparation of Calcium Standard

Volume Extraction Buffer (µL)	Volume Calcium Stock (µL)	Final Concentration (µg)
50	0	Blank
48	2	0.4
46	4	0.8
44	6	1.2
42	8	1.6
40	10	2.0
30	20	4.0

For the original protocol, the calcium samples were dissolved in 50µL of 1x TE buffer. For the new protocol, 55µL of sample was extracted before the acid hydrolysis and plated. For both protocols, each well (sample and standard) received 60µL of calcium assay buffer and 90µL of chromogen. The plate was covered with foil and incubated at room temperature for 10 minutes. The absorbance was read at 575nm (see Figure 11).

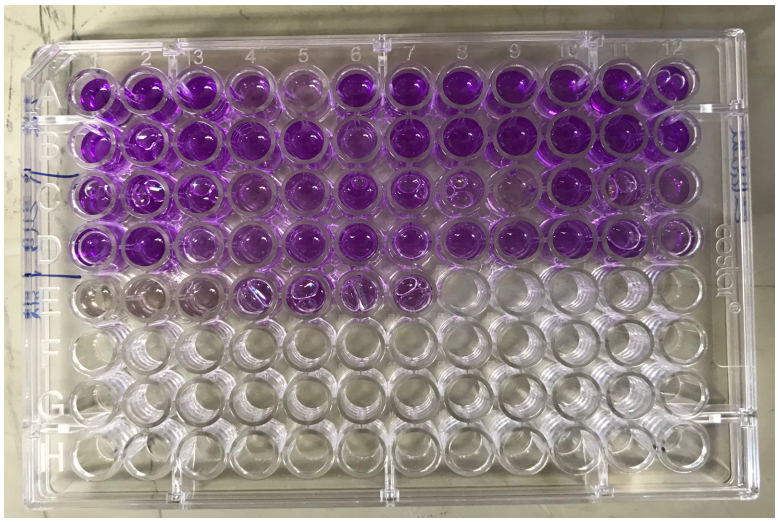

Figure 11. Calcium assay with two patients' samples and standard

Hydroxyproline assay. The hydroxyproline assays were performed on clear 96-well plates following acid hydrolysis using commercially available Sigma-Aldrich Hydroxyproline Assay kits. A standard curve was made by diluting 10µL of the 1mg/mL standard solution with 90µL of ultrapure water, yielding a 0.1mg/mL standard solution. The diluted standard solution was added to individual wells of the plate in varying amounts (0, 2, 4, 6, 8, and 10µL) to generate 0, 0.2, 0.4, 0.6, 0.8, and 1µg/well standards. After the standard curve was made, 100µL of Chloramine T/Oxidation Buffer mixture (6µL Chloramine T concentrate and 94µL oxidation buffer) was added to each sample and standard well, and the plate was incubated at room temperature for 5 minutes. Then, 100µL of diluted DMAB reagent (50µL DMAB concentrate and 50µL perchloric acid/isopropanol) was added to each sample and standard well. The plate was covered with foil (DMAB is color sensitive) and incubated at 60˚C for 90 minutes. The absorbance was read at 560nm (see Figure 12).

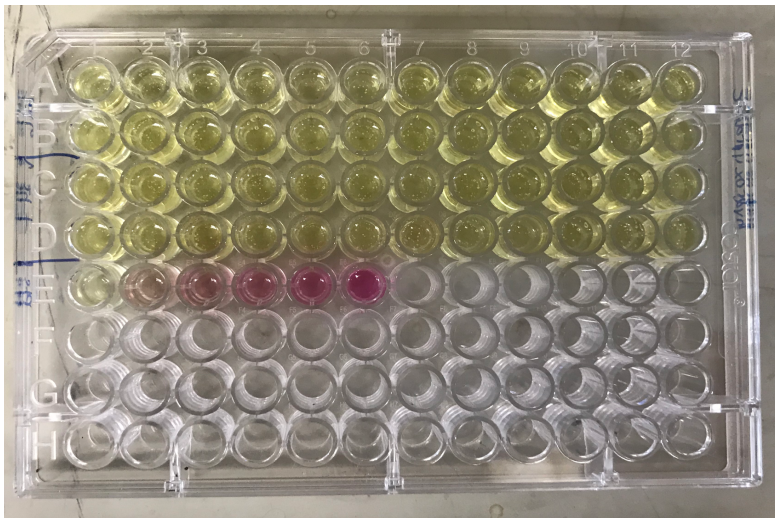

Figure 12. Hydroxyproline assay with two patients' samples and standard

Statistical Analysis

For each assay, the optical density (OD) of the blank was subtracted from the OD of each sample well to correct for background. The blank was also subtracted from the standard OD values and graphed with a line of best fit. The corrected sample values were plugged into the equation for the line of best fit to find the concentration of each well. All concentrations were converted to nanograms, and the concentrations for each assay were matched by well for each patient. The average of the 24 wells was taken to yield each patient's mean ALP, DNA, protein, calcium, and hydroxyproline values. The wells that had no DNA were excluded from the averages for all assays. Only assay data for ALP, DNA, and hydroxyproline were included in the final analyses.

Of the 26 patients in the study, one patient was excluded because the assay wells weren't matched. Another patient was excluded because they had no DNA, making the other assay averages invalid, and one patient's average DNA concentration was an extreme outlier. One patient didn't have any hydroxyproline data due to contamination of

the plate and was thereby excluded. Therefore, final analyses included a total of 22 patients. Data for alizarin nodule growth was not available for three patients, so those analyses only included 19 patients. Additionally, two patients refused to self-identify their race. Due to the low sample size, patients only missing alizarin or race data were included in other analyses not involving those variables. As these were the first statistics involving clinical variables in the study, the analysis plan was primarily exploratory.

RESULTS

Two separate questions were addressed in these studies. The first question related to the reproducibility of the biological assays that were collected for each of the patients. For this set of assessments, the inter-assay coefficient of variability (CV) was measured to assess the assay consistency between plates. This was calculated by finding the mean of the high and low standards and dividing the mean of the means from the standard deviation. It is generally accepted that the inter-assay coefficient of variability (CV) be less than 15% ("Inter- and Intra- Assay Coefficients of Variability", n.d.). The inter-assay CV for the ALP, DNA, and hydroxyproline assays were 3.84% (M = 87.94, SD = 3.37), 23.89% (M = 472.30, SD = 112.84), and 3.70% (M = 0.50, SD = 0.02), respectively. The intra-assay CV was not calculated because the samples run for each participant were not true duplicates. Each of the 24 wells for each patient of each assay were matched to a well on the initial harvesting plate, and each well on any given patient's harvesting plate only correlated with one well (no duplicates) on each assay. Therefore, it would be more appropriate to compare between the different assays rather than within them. The means for each patient's assays were visually inspected to determine how consistently the results clustered (see Figure 13).

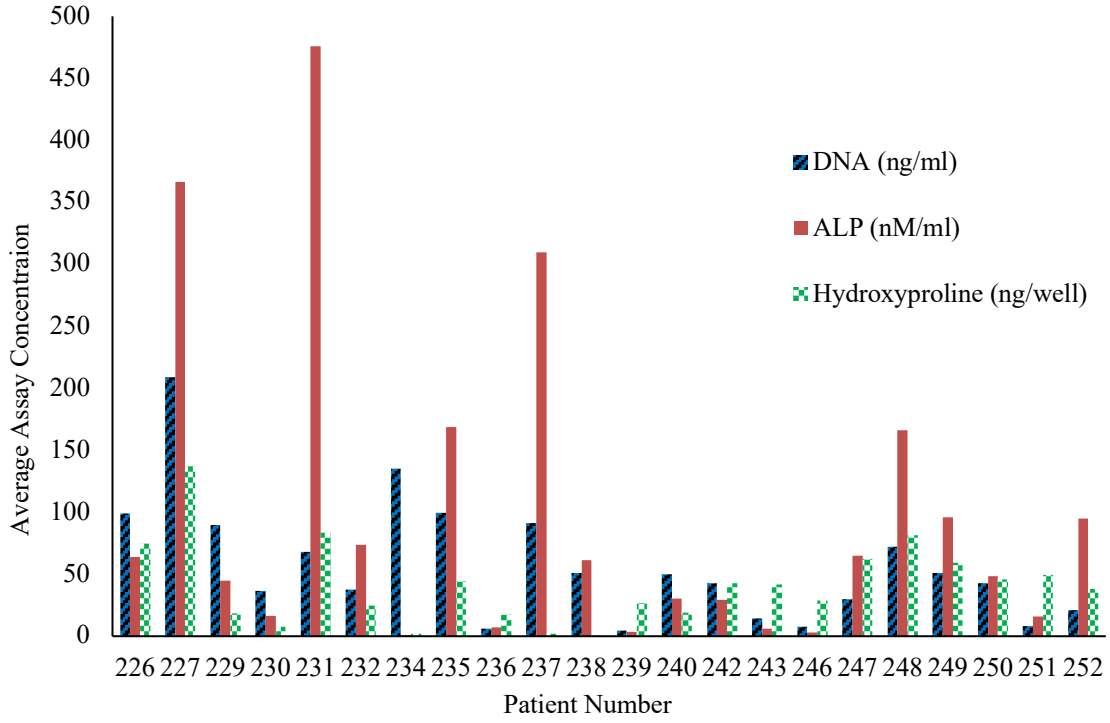

Figure 13. Comparison of average patient assay concentrations

Assay Variables

A Spearman rho correlation was run to determine the linear association between ARS and the assays (ALP, DNA, hydroxyproline). Significance was determined to be a p-value of less than .05. There was a significant positive correlation between ALP and hydroxyproline ($r_s(20) = .469$, $p = .014$) and between ALP and DNA ($r_s(21) = .574$, $p = .002$). This correlation was weaker for hydroxyproline than for DNA. The correlation between DNA and hydroxyproline was not significant. Despite this, the assays were grouped together as the dependent variables in a MANOVA. There was also a significant correlation between alizarin nodules and ALP ($r_s(17) = .685$, $p = .001$). The correlation between alizarin and DNA ($r_s(17) = .315$, $p = .095$) and alizarin and hydroxyproline ($r_s(17) = .310$, $p = .098$) were trending towards significance.

Clinical Variables

The second set of questions that were examined involved the relationship between various biological features that were collected from the various patients' cell cultures to underlying demographic features of the patients from which the cultures were collected. A MANOVA was used to determine the relationship between the clinical factors and the assay concentrations. Significance was determined to be a p-value less than .05. The assays are reported in ng/mL (DNA), nM/mL (ALP), and ng/well (hydroxyproline). While information on 25 different clinical variables were collected for each patient, only main effects or interactions involving sex, age, race, BMI, vitamin D level, renal disease status, and smoker status are presented. The clinical variables were divided into innate factors (sex, age, race) and acquired or variable factors (BMI, smoking status, vitamin D level, renal disease).

Innate factors. A 2x2x3 MANOVA was conducted between the innate factors and the assays. Table 6 summarizes the analyses of variance for ALP and hydroxyproline, with *F* ratios (*df*s given in parentheses), and partial- η^2 values presented for each variable of interest and significant finding. There were no significant findings for DNA (data not shown).

Table 6

ALP and Hydroxyproline MANOVA Results for Innate Factors

	ALP (nM/mL)		Hydroxyproline (ng/well)	
	F	η^2	*F*	η^2
Age (1,11)	0.861	.073	8.007*	.421
Race (2,11)	10.816**	.663	0.390	.066
Sex x Age (1,11)	37.853**	.775	3.800	.257
Sex x Age x Race (1,11)	16.743**	.604	0.062	.006

Note. Partial η^2 indicates the proportion of variance accounted for by each factor.
* $p < .05$, ** $p < .01$

As shown in Table 5, there was a significant main effect of age on hydroxyproline concentration such that patients under 50 years old had significantly more hydroxyproline (*M* = 59.777, *SE* = 10.760) than patients that were 50 years and older (*M* = 24.221, *SE* = 8.488). There was also a main effect of race on ALP. Specifically, patients who self-identified as "white" had more ALP (*M* = 200.774, *SE* = 21.740) than those that identified as "other" (*M* = 94.720, *SE* = 57.972) and "African American" (*M* = 53.232, *SE* = 23.667). Post hoc tests are used to determine which differences were significant, however this could not be done because one of the race groups ("other") had less than two patients included in the analyses. There was a significant three-way interaction between sex, age, and race for ALP concentration. Follow-up analyses were not conducted because one of the groups ("other") had less than two cases, and the other variables had less than two groups. Therefore, the means were presented in a graph to identify the possible differences (see Figure 14). White males under 50 years old had higher ALP concentrations (*M* = 47.505, *SE* = 40.993) than African American males

under 50 (*M* = 2.940, *SE* = 57.972). There were no males under 50 that self-identified as "other." White males 50 years old and showed an increased ALP concentration (*M* = 309.510, *SE* = 57.972) compared to African Americans (*M* = 76.715, *SE* = 23.667) and other races (*M* = 94.720, *SE* = 57.972). For females under 50 years old, those that identified as white demonstrated a greater ALP concentration (*M* = 421.253, *SE* = 40.993) than those that identified as African American (*M* = 95.970, *SE* = 57.972). However, white females 50 years and older had decreased ALP (*M* = 24.827, *SE* = 28.986) than African American females (*M* = 37.305, *SE* = 40.993).

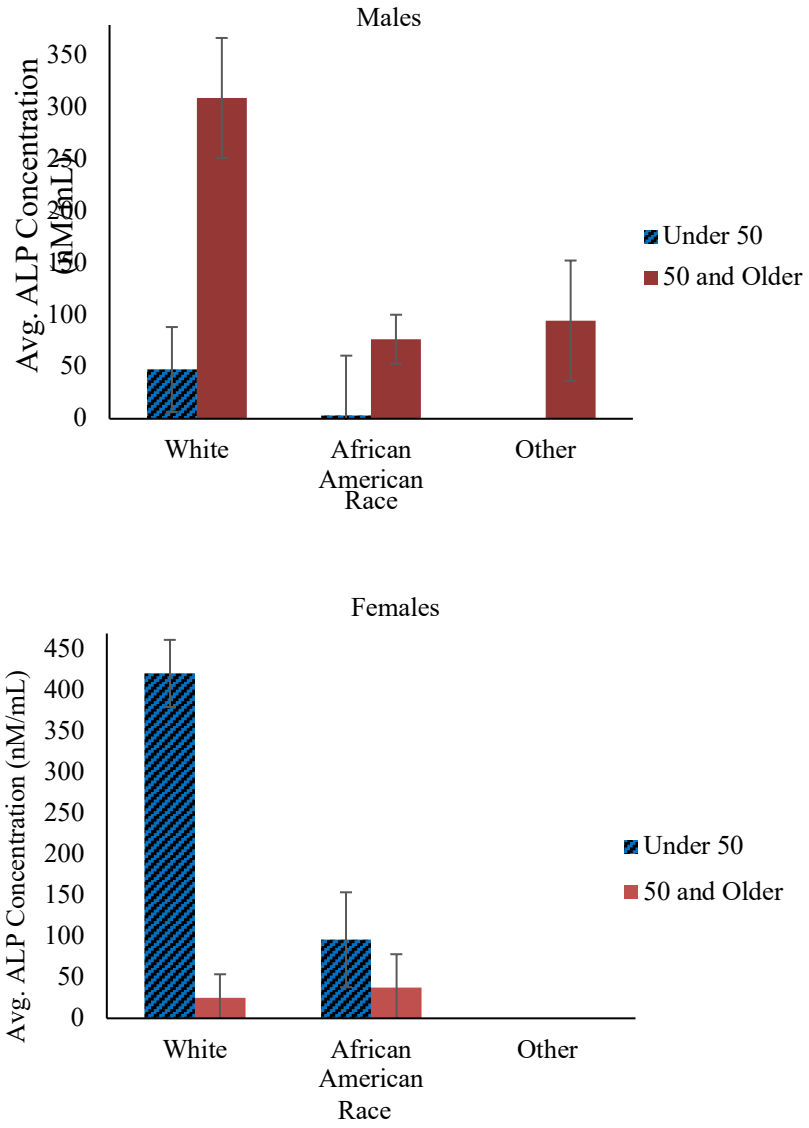

Figure 14. ALP concentration based on age, race, and sex

Acquired factors. One-way MANOVAs were run for BMI, smoker status, vitamin D level, and renal disease status. No significant variables were found. Additional exploratory analyses were run to explore whether any other relationships across variables existed; a series of 2x2 MANOVAs were run between all the variables. Statistics with renal disease status were limited due to the homogeneity of the patient sample with

confirmed renal disease. There was a trend towards significance for the interaction between BMI and smoker status for hydroxyproline ($F(1, 16) = 3.046$, $p = .076$, $\eta^2 = .276$). Specifically, patients with a healthy weight who never smoked before had greater hydroxyproline concentrations *(*$M = 65.704$, $SE = 13.964$) than those who currently smoked ($M = 43.475$, $SE = 22.079$) and those that used to smoke ($M = 25.263$, $SE = 18.028$). Unlike the healthy group, the patients that never smoked and were overweight had less hydroxyproline concentrations on average ($M = 1.190$, $SE = 22.079$) than those that currently smoked and used to smoke (see Figure 15). Overweight patients that used to smoke ($M = 43.637$, $SE = 11.040$) had similar hydroxyproline concentrations to those that were overweight and currently smoked ($M = 32.390$, $SE = 22.079$). None of the other MANOVA analyses were significant.

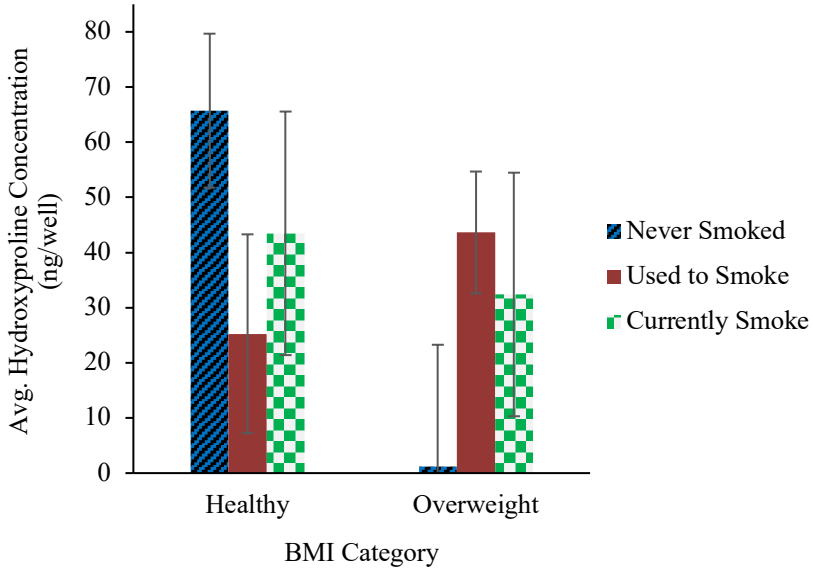

Figure 15. Hydroxyproline concentration by smoker status and BMI

DISCUSSION

Assay Reliability

The reproducibility of the assays was determined by calculating the inter-assay CVs. It was shown for ALP, DNA, and hydroxyproline that the assays were all well below the recommended threshold of 15%, indicating that plate-to-plate variation was fairly low ("Inter- and Intra- Assay Coefficients of Variability, n.d.). In addition, the relationship between the biochemical markers were determined. Patients with more mineral formation, as measured by ARS, were expected to have greater DNA, ALP, and hydroxyproline concentrations. It was shown that there was a positive correlation between ARS and the assay variables. Although the ARS and DNA and ARS and hydroxyproline correlations weren't significant, they were trending. With additional patients, these findings may be significant. Additionally, future analyses could be more accurate by extracting and assaying the ARS dye opposed to visual inspection and ranking.

It was hypothesized that the patients with the higher concentrations of DNA would have the greatest concentrations of ALP and hydroxyproline. The correlations showed significance for ALP and hydroxyproline and ALP and DNA. However, the correlation between DNA and hydroxyproline was not significant. This may be due to the small sample size. Despite this, all three assays were grouped together for the subsequent MANOVAs. It should be noted that the results should be interpreted with caution. Visual inspection of the assay means supported the correlational findings. Specifically, patients with decreased ALP generally also showed decreased DNA and hydroxyproline

compared to patients with increased ALP concentrations (see Figure 13). This relationship was less consistent with the increased ALP concentrations, suggesting that patients with greater ALP did not appear to have more DNA or hydroxyproline.

Innate Factors

Age had a significant effect on hydroxyproline demonstrated by the significant increase in hydroxyproline in patients under 50 years old had compared to patients who were 50 years old and over. This is consistent with the literature, supporting that advanced age is associated with greater rates of bone resorption (Rodan & Reszka, 2003). Although age was significant for hydroxyproline, a measure of bone resorption, it was not significant for ALP, a measure of bone deposition. This may indicate that, as one ages, bone resorption outpaces deposition, leading to osteoporosis. Race also had a significant effect on ALP showed by the increased ALP in the cells isolated from the white patients compared to all the other racial groups. This was unexpected given that osteoporosis incidence is seen more in whites than in other races (Looker et al., 2010). Due to the small sample size in each racial group, formal post-hoc tests could not be conducted to determine where the significant differences were. Although there was no significant effect of age on ALP concentration, there was a significant interaction between sex, age, and race. Visual inspection of the means suggested that white males under 50 had greater ALP than African American males under 50. The same relationship was seen for males 50 and over. Although it was expected that bone formation makers, such as ALP, would be decreased in patients who were white due to the increased prevalence of osteoporosis and subsequent fracture risk, research has suggested that the

relationship between osteoporosis rates and age in males is less clearly defined and more random than it is for women (Cummings & Melton, 2002). For females less than 50 years old, ALP was increased in patients who were white compared to patients who were African American. However, for females 50 and over, this was not observed; ALP was actually decreased in whites. Since decreased ALP may be indicative of reduced bone formation, this result is consistent with the literature supporting increased rates of bone loss and osteoporosis in postmenopausal white women relative to postmenopausal African American women and premenopausal women (Rodan & Reszka, 2003; Silverman & Madison, 1988). This result also supported our hypothesis regarding decreased ALP concentration in postmenopausal white women, however there was no concordant significant finding for hydroxyproline. In addition to the small sample size, all but one of the patients self-identified as white or African American. Future analyses with more patients should also include Hispanics, Asians, and other races to get a more comprehensive understanding of the relationship between race and osteoporosis.

Clinical Factors

There were no significant findings for any of the clinical variables, however there was a trend in the relationship between BMI and smoker status for hydroxyproline concentration. Specifically, average hydroxyproline concentration for individuals with a healthy BMI were greater for patients that had never smoked compared to those that currently smoke, and it was lowest for those that used to smoke. Alternately, for overweight BMI patients, the average hydroxyproline amount was decreased for those who never smoked compared to patients who used to smoke or currently smoke.

Although research suggests that smoking causes increased osteolysis and decreased bone formation due to its effects on vitamin D and PTH, these results do not outright support that (Brot, Jorgensen, & Sorensen, 1999). Decreased hydroxyproline for the overweight non-smokers may suggest that there is decreased bone resorption compared to patients that have smoked. The different patterns in hydroxyproline concentrations across smoking groups may indicate that smoking has a more detrimental effect on bone degradation when the patient is also overweight. However, more patients are needed for each group to support this claim. Furthermore, research has supported that being underweight (BMI $\leq 19 kg/m^2$), and other variables related to lower weight (i.e. weight loss, anorexia nervosa, vitamin deficiencies, excessive thinness), are associated with increased bone fragility and fractures (Frost, 1995). None of the patients reported in these analyses had a BMI of 19 or less, therefore this relationship could not be tested in this sample.

Limitations and Future Directions

Given the limitations of this study, all results should be interpreted with caution. Due to the small sample size, some patients were included in certain analyses and excluded from others if data was missing for that patient. Although all of the reported patients met the inclusion criteria, there is a chance with human research that there is some extraneous variable that was not taken into account that affects the results. Although extreme outliers in the data set were excluded, there was a lot of variation in assay concentrations across patients. Some of this variation may be attributed to variations in volume, content, and depth of the reamings from the acetabular coring of

each patient. The actual MSC count plated may have varied across wells, too. During seeding, MSCs were plated from the filtered bone marrow cell solution based on the cell count. Nothing was done to separate the red blood cells, which made up the majority of the solution, from the MSCs. Further studies could mitigate this issue by using integrating Ficoll-Paque and RBC lysis treatments into the MSC processing protocol (Horn et al., 2008). There was also a certain degree of homogeneity across the patients. As a result, some comparisons had groups with only one or two patients. Future studies should include more patients evenly dispersed across the grouping variables. Lastly, self-report bias may affect some of the findings since certain variables, like race and smoking status, were reported by the patients.

This study examined the relationship between various osteoporosis risk factors and biochemical markers of bone turnover. The goals were to correlate the biochemical markers and identify any relationships between the markers and the risk factors. Our findings demonstrate that postmenopausal white females may have a greater risk for osteoporosis than premenopausal females and females of different races due to decreased bone deposition, as measured by ALP concentrations. Although there were no significant findings for the acquired variables, there were some interesting trends for BMI and smoking status that should be further explored in future studies. Osteoporosis affects more than 25 million people and accounts for upwards of 1.5 million fractures every year in the US alone. Understanding the mechanical and biochemical processes underlying osteoporosis, particularly in regard to genetics, will help with the diagnosis and treatment

of osteoporosis as well as the prevention of osteoporosis related fractures (Dobbs, Buckwalter, & Saltzman, 1999).

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